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(54) Title: YEAST STRAINS

(57) Abstract

Reduction (preferably elimination) of the HSP150 protein in a yeast used to produce desired foreign proteins, especially human albumin, facilitates subsequent purification of the protein.

5' and 3' regions of *HSP150* gene obtained by PCR:

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YEAST STRAINS

Field of the Invention

5   The present invention relates to the production of heterologous proteins by
yeast species and more particularly to an adaptation of the yeast in which the
protein is produced.

Background and Prior Art

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In recent years, yeasts have been widely used as host organisms for the
production of heterologous proteins (reviewed by Romanos *et al*, 1992),
including recombinant human albumin (rHA) (Sleep *et al*, 1990, 1991; Fleer
et al, 1991).   Yeasts are readily amenable to genetic manipulation, can be
15   grown to high cell density on simple media, and as eukaryotes are suitable for
the production of secreted as well as cytosolic proteins.

When yeasts are utilised to produce a desired heterologous protein by secretion
into the growth medium, a large number of host-derived proteins may also be
20   present, including other proteins secreted by the host but also intracellular
proteins present in the supernatant as the result of leakage from cells or cell
lysis. In processes in which the protein is not secreted, there is of course an
even higher level of contamination with intracellular yeast proteins.   It is
necessary to purify the desired protein and to remove these contaminating
25   proteins from the preparation; such methods have been disclosed in WO
92/04367 and EP 524 681. The majority of contaminating proteins will have
physicochemical properties sufficiently different from the desired protein to
permit efficient separation by standard techniques, such as ion exchange or size
exclusion chromatography.   The prior art gives the impression that such
30   proteins can be satisfactorily removed by such techniques; see, for example EP

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524 681 (Gist-brocades), EP 570 916 (Green Cross) and EP 464 590 (Green Cross). Indeed, we have developed sophisticated chromatographic techniques (unpublished) to remove contaminating proteins from desired proteins.

5   **Summary of the Invention**

We have now also adopted a different approach and have identified the gene responsible for a protein, namely the *HSP150* gene, which co-purifies with recombinant human albumin (rHA) and, in principle, with other desired

10   proteins. In accordance with the invention, we eliminate the contaminating protein from the initial fermentation, rather than develop highly sophisticated and complex means of removal during purification. This protein was not previously known to be a co-purifying contaminant.

15   In one aspect of the invention, the *HSP150* gene is functionally deleted from the genome of the host. This has not caused any detrimental effects on production of the desired protein and removes a potential contaminant that has proven difficult to remove by standard purification techniques. Despite the presence of at least two closely related genes encoding proteins very similar to

20   Hsp150, *PIR1* and *PIR3*, in such modified yeast, rHA purified from these organisms does not contain detectable levels of any protein from this family.

The *S. cerevisiae* Hsp150 protein was originally described by Russo *et al* (1992) and was shown to be produced constitutively, to be extensively O-

25   glycosylated and to be secreted efficiently into the growth medium. A 7-fold increase in the level of Hsp150 protein was seen when cells grown at 28°C were shifted to 37°C. Makarow has proposed preparing fusions of Hsp150 (or fragments thereof) and a desired protein, in order to achieve enhanced, controllable secretion (WO 93/18167). The *HSP150* gene encodes a primary

30   translation product of 413 amino acids, including an N-terminal secretion signal

sequence of 18 amino acids that is not present in the mature protein. A further post-translational processing event occurs C-terminal to a pair of basic residues to yield two subunits of 54 and 341 amino acids which remain associated. The 341 amino acid subunit contains 11 tandem repeats of a 19 amino acid

5   sequence, the function of which is unknown. Homologues of the *HSP150* gene were found in *Torulaspora delbrueckii*, *Kluyveromyces marxianus* and *Schizosaccharomyces pombe* (Russo *et al*, 1992).

The same protein has been designated the PIR2 protein by Toh-e *et al* (1993).

10 The *HSP150/PIR2* gene was shown to be a member of a family of at least three genes *(PIR1, PIR2* and *PIR3)* all of which contain similar internal tandem repeats of approximately 19 amino acids. Homologues of the *PIR* genes were shown to be present also in *Kluyveromyces lactis* and *Zygosaccharomyces rouxii* (Toh-e *et al*, 1993). Disruption of the *HSP150/PIR2* gene showed that

15 this is not an essential gene (Russo *et al*, 1992; Toh-e *et al*, 1993).

In this specification we refer to rHA as the desired protein. However, it is to be understood that the problem addressed by the invention will, in principle, be encountered with any other protein which has similar properties to those of

20 rHA and which is therefore purified in the same way. Thus, the solution provided by the invention, namely elimination of Hsp150, is applicable also to the production of such other proteins.

Our studies have revealed that the Hsp150 protein is inefficiently separated

25 from rHA by ion exchange chromatography. Surprisingly, however, Hsp150 does not appear in the fraction equivalent to the rHA fraction when rHA is absent. For example, when rHA-containing culture supernatant is passed through a cation exchange column under conditions which ensure binding of the rHA to the column (eg pH4.5, conductivity <7mS), Hsp150 also binds to the

30 column and is eluted under the same conditions as rHA and thus contaminates

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the rHA preparation. However, when culture supernatant from a yeast that does not secrete rHA is passed through such a column under the same conditions, the Hsp150 protein does not bind to the matrix but passes straight through the column. The eluate fraction does not contain Hsp150 in the

5   absence of rHA. Similarly, the Hsp150 protein does not bind to an anion exchange column run under conditions which would result in binding of albumin (eg pH5.5, 1.5mS) in the absence of rHA, but is present in the rHA eluate fraction when rHA is present. Surprisingly, we have found that the presence of rHA in culture supernatant significantly alters the behaviour of

10   some yeast proteins during chromatographic purification of the rHA such that proteins with physico-chemical properties which indicate that they would be separated from albumin by, for instance, ion exchange chromatography in fact contaminate the rHA preparation and are difficult to remove.

15   One aspect of the invention provides a process for preparing a desired protein from yeast, comprising culturing the yeast and obtaining the protein, characterised in that the yeast is deficient in heat shock protein 150 (Hsp150).

The most convenient way of achieving this is to create a yeast which has a

20   defect in its genome such that a reduced level of the Hsp150 protein is produced. For example, there may be a deletion, insertion or transposition in the coding sequence or the regulatory regions (or in another gene regulating the expression of the Hsp150 gene) such that little or no Hsp150 protein is produced. Alternatively, the yeast could be transformed to produce an anti-

25   Hsp150 agent, such as an anti-Hsp150 antibody.

To modify the *HSP150* gene so that a reduced level of co-purifying protein is produced, site-directed mutagenesis or other known techniques can be employed to create single or multiple mutations, such as replacements, insertions,

30   deletions, and transpositions, as described in Botstein and Shortle, "Strategies

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and Applications of *In Vitro* Mutagenesis", *Science*, **229:** 193-210 (1985), which is incorporated herein by reference. Suitable mutations include chain termination mutations (clearly stop codons introduced near the 3' end might have insufficient effect on the gene product to be of benefit; the person skilled

5   in the art will readily be able to create a mutation in, say, the 5' three quarters of the coding sequence), point mutations that alter the reading frame, small to large deletions of coding sequence, mutations in the promoter or terminator that affect gene expression and mutations that de-stabilize the mRNA. Some desirable point mutations or specific amino acid substitutions may affect

10   chromatographic behaviour by altering the charge distribution. Hence, the protein produced has a similar primary amino acid sequence to that of native Hsp150, but is functionally distinct such that it will not co-purify with the desired protein. Such a modified protein is not regarded as being Hsp150. Specific mutations can be introduced by an extension of the gene disruption

15   technique known as gene transplacement (Winston, F. *et al* (1983) *Methods Enzymol.* **101,** 211-228).

Any polypeptides inserted into the Hsp150 protein should not be, and should not create, ligands for the desired protein. Those skilled in the art can readily

20   determine, by simple binding assays, whether a ligand has been used or created. Generally one uses a selectable marker to disrupt a gene sequence, but this need not be the case, particularly if one can detect the disruption event phenotypically. In many instances the insertion of the intervening sequence will be such that a stop codon is present in frame with the Hsp150 sequence and the

25   inserted coding sequence is not translated. Alternatively the inserted sequence may be in a different reading frame to Hsp150.

The gene may have one or more portions (optionally including regulatory regions, up to the whole gene) excised or inverted, or it may have a portion

30   inserted, in order to result either in no production of protein from the *HSP150*

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locus or in the production of protein from the *HSP150* locus which does not co-purify with the desired protein.

Preferably, the yeast secretes the desired protein, which is then purified from the fermentation medium. The purification may take place elsewhere; hence, production of culture medium, containing desired protein, in which the level of Hsp150 protein is low or zero is an end in itself.

A protein is generally regarded as co-purifying with Hsp150 if the two are still associated after two dissimilar chromatographic separation techniques (one of which is affinity chromatography for the desired protein) or, if affinity chromatography is not used, if the proteins are still associated after three dissimilar steps (for example an anion exchange, a cation exchange and a gel permeation step). Essentially, the identity of the desired protein is self-defined: if a person skilled in the art finds that his desired protein is, after an otherwise suitable purification process, contaminated with a yeast protein, he can determine (using known methods, which are explained in more detail below) whether that yeast protein is Hsp150 and, if it is, use the yeasts and methods of the invention; if the desired protein is not contaminated with Hsp150, then the need for the present invention will not arise. We have found the process of the invention to be particularly applicable to albumins and to other proteins which have similar physico-chemical properties to albumins, such that they are purified by similar chromatographic techniques. Preferably, the desired protein is a human albumin.

Human serum albumin (HSA) is a protein of 585 amino acids that is present in human serum at a concentration of 35-45g L^{-1} and represents about 60% of the total serum protein. HSA is responsible for a significant proportion of the osmotic pressure of serum, and also functions as a carrier of endogenous and exogenous ligands. It is used clinically in the treatment of patients with severe

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burns, shock, or blood loss, and at present is produced commercially by extraction from human blood. The production of recombinant human albumin (rHA) in microorganisms has been disclosed in EP 330 451 and EP 361 991.

5　The albumin may be a variant of normal HSA/rHA. By "variants" we include insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the oncotic, useful ligand-binding or non-immunogenic properties of albumin. In particular, we include naturally-occurring polymorphic variants of human albumin; fragments of human

10　albumin, for example those fragments disclosed in EP 322 094 (namely HSA (1-n), where n is 369 to 419); and fusions of albumin with other proteins, for example the kind disclosed in WO 90/13653.

By "conservative substitutions" is intended swaps within groups such as Gly,

15　Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

A second main aspect of the invention provides a yeast transformed to express a desired protein which will co-purify with Hsp150 in chromatographic techniques, characterised in that the yeast is deficient in such Hsp150.

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In addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

25

The desired protein is produced in conventional ways, for example from a coding sequence inserted in the yeast chromosome or on a free plasmid.

The yeasts are transformed with a coding sequence for the desired protein in

30　any of the usual ways, for example electroporation. Methods for

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transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

5   Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct can be grown to produce the desired polypeptide. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985)
10   *Biotech.* **3**, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies.

Useful yeast plasmid vectors include pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037,
15   USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

20   A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.
25

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that
30   remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic

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activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA
segments. The blunt-ended segments are then incubated with a large molar
5   excess of linker molecules in the presence of an enzyme that is able to catalyze
the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA
ligase. Thus, the products of the reaction are DNA segments carrying
polymeric linker sequences at their ends. These DNA segments are then
cleaved with the appropriate restriction enzyme and ligated to an expression
10   vector that has been cleaved with an enzyme that produces termini compatible
with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are
commercially available from a number of sources including International
15   Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA in accordance with the invention is to use
the polymerase chain reaction as disclosed by Saiki et al (1988) Science 239,
487-491. In this method the DNA to be enzymatically amplified is flanked by
20   two specific oligonucleotide primers which themselves become incorporated
into the amplified DNA. The said specific primers may contain restriction
endonuclease recognition sites which can be used for cloning into expression
vectors using methods known in the art.

25   Any yeast which produces an Hsp150 protein can be modified in accordance
with the invention. Exemplary genera of yeast contemplated to be useful in the
practice of the present invention are Pichia (Hansenula), Saccharomyces,
Kluyveromyces, Candida, Torulopsis, Torulaspora, Schizosaccharomyces,
Citeromyces, Pachysolen, Debaromyces, Metschunikowia, Rhodosporidium,
30   Leucosporidium, Botryoascus, Sporidiobolus, Endomycopsis, and the like.

10

Preferred genera are those selected from the group consisting of *Saccharomyces*, *Schizosaccharomyces*, *Kluyveromyces*, and *Torulaspora*. Examples of *Saccharomyces* spp. are *S. cerevisiae*, *S. italicus* and *S. rouxii*. Examples of *Kluyveromyces* spp. are *K. fragilis*, *K. lactis* and *K. marxianus*.

5   A suitable *Torulaspora* species is *T. delbrueckii*. Examples of *Pichia* *(Hansenula)* spp. are *P. angusta* (formerly *H. polymorpha*), *P. anomala* (formerly *H. anomala*) and *P. pastoris*.

Homologues of *HSP150* have already been shown to be present in a wide range

10   of different yeast genera: *Torulaspora* sp., *Kluyveromyces* sp., *Schizosaccharomyces* sp. and *Zygosaccharomyces* sp. (Russo *et al*, 1992; Toh-e *et al*, 1993). In addition, our own studies have shown by Southern blotting that *Pichia* sp. possess a homologue of *HSP150*.

15   Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

Suitable promoters for *S. cerevisiae* include those associated with the *PGK1*

20   gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α-mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GUT2* promoter, the *GPD1*

25   promoter, and hybrid promoters involving hybrids of parts of 5' regulatory regions with parts of 5' regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

Convenient regulatable promoters for use in *Schizosaccharomyces pombe* are

30   the thiamine-repressible promoter from the *nmt* gene as described by Maundrell

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(1990) *J. Biol. Chem.* **265**, 10857-10864 and the glucose-repressible *fbp1* gene promoter as described by Hoffman & Winston (1990) *Genetics* **124**, 807-816.

Methods of transforming *Pichia* for expression of foreign genes are taught in,
5   for example, Cregg *et al* (1993), and various Phillips patents (eg US 4 857 467, incorporated herein by reference), and *Pichia* expression kits are commercially available from Invitrogen BV, Leek, Netherlands, and Invitrogen Corp., San Diego, California. Suitable promoters include *AOX1* and *AOX2*.

10   The Gellissen *et al* (1992) paper mentioned above and Gleeson *et al* (1986) *J. Gen. Microbiol.* **132**, 3459-3465 include information on *Hansenula* vectors and transformation, suitable promoters being *MOX1* and *FMD1*; whilst EP 361 991, Fleer *et al* (1991) and other publications from Rhône-Poulenc Rorer teach how to express foreign proteins in *Kluyveromyces* spp., a suitable promoter being
15   *PGK1*.

The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be
20   those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae ADH1* gene is preferred.

The desired protein may be initially expressed with a secretion leader sequence,
·25 which may be any leader effective in the yeast chosen. Leaders useful in *S. cerevisiae* include that from the mating factor α polypeptide (MFα-1) and the hybrid leaders of EP-A-387 319. Such leaders (or signals) are cleaved by the yeast before the mature albumin is released into the surrounding medium. Further such leaders include those of *S. cerevisiae* invertase (*SUC2*) disclosed
30   in JP 62-096086 (granted as 91/036516), acid phosphatase (*PHO5*), the pre-

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sequence of MFα-1, β-glucanase (*BGL2*) and killer toxin; *S. diastaticus* glucoamylase II; *S. carlsbergensis* α-galactosidase (*MEL1*); *K. lactis* killer toxin; and *Candida* glucoamylase.

5   **Detailed Description of the Invention**

Preferred aspects of the invention will now be described in more detail, with reference to the accompanying drawings, in which

10   Figure 1 is a scheme showing the preparation of an *Eco*RI *HSP150-URA3-HSP150* fragment used to transform a yeast strain (DBU3) and disrupt the *HSP150* gene (Example 1); and

Figure 2 is a scheme showing the preparation of a further *Eco*RI fragment used
15   to remove the *HSP150* coding sequence altogether (Example 2).

All standard recombinant DNA procedures are as described in Sambrook *et al* (1989) unless otherwise stated. The DNA sequences encoding rHA are derived from the cDNA disclosed in EP 201 239.
20

Example 1

The *HSP150* gene was mutated by the process of gene disruption (Rothstein, 1983) which effectively deleted part of the *HSP150* coding sequence, thereby
25   preventing the production of Hsp150.

Four oligonucleotides suitable for the PCR amplification of the 5' and 3' ends of the *HSP150* gene (Russo *et al*, 1992) were synthesized using an Applied Biosystems 380B Oligonucleotide Synthesizer.
30

5' End

LRE45:   5'-CTATTTCCTATTTCGGGAATTCTTAAAGACAAAAAAGCTC-3'

LRE46:   5'-GGCTGTGGTGCTGCAGATGATGCGCTGG-3'

5   **3' End**

LRE47:   5'-GCTACTTCCGCTTCTGCAGCCGCTACCTCC-3'

LRE48:   5'-GCCGTGTAGCGAGGGAATTCTGTGGTCACGATCACTCG-3'

Note, LRE45 and LRE48 contain changes in the *HSP150* gene sequence so as

10   to introduce *Eco*RI sites into the 5' or the 3' end of the *HSP150* gene PCR

products. LRE46 and LRE47 both contain *Pst* I sites naturally present in the

HSP150 gene sequence (SEQ 1).

PCR was carried out to amplify individually the 5' and 3' ends of the *HSP150*

15   gene, using LRE45 and LRE46 or LRE47 and LRE48 respectively, from the

DNA from *S. cerevisiae* genomic DNA (Clontech Laboratories, Inc.).

Conditions were as follows: 1μg/ml genomic DNA, $\simeq 1.2 \times 10^{-10}$ moles of each

primer, denature at 94°C for 61 seconds, anneal at 37°C for 121 seconds,

20   DNA synthesis at 72°C for 181 seconds for 30 cycles, with a 10 second

extension to the DNA synthesis step after each cycle, followed by a 4°C soak.

PCR was carried out using a Perkin-Elmer-Cetus Thermal cycler and a Perkin-

Elmer-Cetus PCR kit was used according to the manufacturer's

recommendations. PCR products were analysed by gel electrophoresis and

25   were found to be of the expected size. Each PCR product was digested with

*Eco*RI and *Pst*I and cloned into *Eco*RI/*Pst*I digested pUC19 (Yanisch-Perron *et*

al, 1985) to form pAYE503 (containing the 5' end of the *HSP150* gene) and

pAYE504 (containing the 3' end of the *HSP150* gene) (see Fig. 1).

30   Plasmid DNA sequencing was carried out on pAYE503 and pAYE504 to

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confirm that the inserts were the desired sequences. pAYE503 and pAYE504 were digested with *Eco*RI and *Hin*dIII and the *HSP150* gene fragments were isolated and cloned together into pUC19XH (a derivative of pUC19 lacking a *Hin*dIII site in its polylinker) to form pAYE505. The *URA3* gene was isolated

5   from YEp24 (Botstein *et al*, 1979) as a *Hin*dIII fragment and cloned into the *Hin*dIII site of pAYE505 to form pAYE506 (Fig. 1). pAYE506 contains a selectable marker (*URA3*) flanked by 5' and 3' regions of the *HSP150* gene.

To construct a strain lacking the capacity to produce HSP150, a *ura3* derivative

10   of DB1 cir° pAYE316 (Sleep *et al*, 1991) was obtained by random chemical mutagenesis and selection for resistance to 5-fluoro-orotic acid (Boeke *et al*, 1987). Plasmid pAYE316 is based on the 2 μm plasmid and contains a coding sequence for human albumin under the control of the yeast *PRB1* promoter, with an *ADH1* terminator and a *LEU2* selectable marker.

15

The strain was grown overnight in 100mL buffered minimal medium (Yeast Nitrogen Base [without amino acids, without ammonium sulphate, Difco], $(NH_4)_2SO_4$ 5g/L, citric acid monohydrate 6.09g/L, NaHPO$_4$ 20.16g/L, sucrose 20g/L, pH6.5) and the cells were collected by centrifugation and then washed

20   once with sterile water. The cells were then resuspended in 10mL sterile water and 2mL aliquots were placed in separate 15mL Falcon tubes. A 5mg/mL solution of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was then added to the tubes as follows: 0μL, 20μL, 40μL, 80μL or 160μL. The cells were then incubated at 30°C for 20 min and then centrifuged and washed three times with

25   sterile water. Finally, the cells were resuspended in 1mL YEP (1%$^w/_v$ yeast extract, 2%$^w/_v$ Bacto peptone) and stored at 4°C. The percentage of cells that survived the mutagenic treatment was determined by spreading dilutions of the samples on YEP plates containing 2%$^w/_v$ sucrose and incubating at 30°C for 3 days. Cells from the treatment which gave approximately 50% survival were

30   grown on YEP plates containing 2%$^w/_v$ sucrose and then replica-plated onto

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YNB minimal medium containing 2%^{w}/$_v$ sucrose and supplemented with 5-fluoro-orotic acid (1mg/mL) and uracil (50μg/mL). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in the absence of uracil supplementation and that this defect could be corrected by

5   introduction of the URA3 gene by transformation.

The ura3 strain, DBU3 cir° (pAYE316), was transformed with EcoRI digested pAYE506 and Ura$^+$ transformants were selected. The disruption of the HSP150 gene in these transformants was confirmed by Southern blot analysis

10   using a fragment comprising the 5' and 3' ends of the HSP150 gene (the EcoRI fragment from pAYE505) as a probe.

The yeast was then grown to high cell density by fed batch culture in minimal medium in a fermenter (Collins, 1990). Briefly, a fermenter of 10L working

15   volume was filled to 5L with an initial batch medium containing 50 mL/L of a concentrated salts mixture (Table 1), 10 mL/L of a trace elements solution (Table 2), 50 mL/L of a vitamins mixture (Table 3) and 20 g/L sucrose. An equal volume of feed medium containing 100 mL/L of the salts mixture, 20 mL/L of the trace elements mixture, 100 mL/L of vitamins solution and 500

20   g/L sucrose was held in a separate reservoir connected to the fermenter by a metering pump. The pH was maintained at 5.7 $\pm$ 0.2 by the automatic addition of ammonium hydroxide or sulphuric acid, and the temperature was maintained at 30°C. The stirrer speed was adjusted to give a dissolved oxygen tension of >20% air saturation at 1 v/v/min air flow rate.

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Table 1. Salts Mixture

Chemical	Concentration (g/L)
KH_2PO_4	114.0
$MgSO_4$	12.0
$CaCl_2.6H_2O$	3.0
Na_2EDTA	2.0

Table 2. Trace Elements Solution

Chemical	Concentration (g/L)
$ZnSO_4.7H_2O$	3.0
$FeSO_4.7H_2O$	10.0
$MnSO_4.4H_2O$	3.2
$CuSO_4.5H_2O$	0.079
H_3BO_3	1.5
KI	0.2
$Na_2MoO_4.2H_2O$	0.5
$CoCl_2.6H_2O$	0.56
H_3PO_4	75mL/L

Table 3. Vitamins Solution

Chemical	Concentration (g/L)
Ca pantothenate	1.6
Nicotinic acid	1.2
m-inositol	12.8
Thiamine HCl	0.32
Pyridoxine HCl	0.8
Biotin	0.008

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The fermenter was inoculated with 100 mL of an overnight culture of *S. cerevisiae* grown in buffered minimal medium (Yeast nitrogen base [without amino acids, without ammonium sulphate, Difco] 1.7 g/L, $(NH_4)_2SO_4$ 5 g/L, citric acid monohydrate 6.09 g/L, Na_2HPO_4 20.16 g/L, sucrose 20 g/L,

5   pH6.5). The initial batch fermentation proceeded until the carbon source had been consumed, at which point the metering pump was switched on and the addition of feed was computer controlled (the micro MFCS system, B. Braun, Melsungen, Germany) using an algorithm based on that developed by Wang *et al* (1979). A mass spectrometer was used in conjunction with the computer

10   control system to monitor the off gases from the fermentation and to control the addition of feed to maintain a set growth rate (eg 0.1 h^{-1}). Maximum conversion of carbon substrate into biomass is achieved by maintaining the respiratory coefficient below 1.2 (Collins, 1990) and, by this means, cell densities of approximately 100 g/L cell dry weight can be achieved.

15

The fermentation broth was centrifuged to remove the cells and then subjected to affinity chromatographic purification as follows. The culture supernatant was passed through a Cibacron Blue F3GA Sepharose column (Pharmacia) which was then washed with 0.1M phosphate glycine buffer, pH8.0. The rHA

20   was then eluted from the column with 2M NaCl, 0.1M phosphate glycine, pH8.0. The albumin may alternatively be purified from the culture medium by any of the variety of known techniques for purifying albumin from serum or fermentation culture medium, for example those disclosed in WO 92/04367, Maurel *et al* (1989), Curling (1980) and EP 524 681.

25

Analysis of rHA purified from *Hsp150* strains revealed that no HSP150 protein was present in these samples. HSP150 protein is determined using prior art techniques such as ELISA or Western blotting.

30   Anti-HSP150 antibodies are disclosed in Russo *et al* (1992) *Proc. Nat. Acad.*

18

Sci. (USA) **89**, 3671-3675.

Example 2

5   The *HSP150* protein coding sequence was deleted by using alternative fragments of the cloned *HSP150* sequences as follows.

The *URA3 Hind*III fragment from YEp24 (see Example 1) was cloned into pIC19R (Marsh J.L. *et al* (1984) *Gene* **32**, 481-485) at *Hind*III to form

10   pAYE601 and then excised as a *SalI/ClaI* fragment and inserted into pAYE505 at the *XhoI* and *ClaI* sites to form pAYE602 (Fig 2). This plasmid was digested with *EcoRI* and then used to transform DBU3 cir° (pAYE316), selecting for Ura$^+$ transformants. The disruption of the *HSP150* gene in these transformants was confirmed by Southern blot analysis as described in Example

15   1.

Thus, in this example, the whole of the *HSP150* coding sequence is removed, whereas in Example 1 the sequence is disrupted to yield non-functional protein.

20   ## Example 3

Southern blotting has revealed an Hsp150 homologue in *Hansenula polymorpha* (now called *Pichia angusta*). The *P. angusta* gene may be functionally deleted by ways analogous to those in Examples 1 and 2.

25

19

References

Boeke, J. D. *et al* (1987) *Methods Enzymol.* **154**, 164-175.

5   Botstein, D. *et al* (1979) *Gene* **8**, 17-24.

Collins, S.H. (1990) <u>In</u> Protein Production by Biotechnology (Harris, T.J.R., ed.) pp 61-77, Elsevier, Barking, Essex.

10   Curling (1980) "Albumin Purification by Ion Exchange Chromatography", in "Methods of Plasma Protein Purification", Ed. Curling, J.M., Academic Press, London.

Fleer, R. *et al* (1991) *Bio/Technology* **9**, 968-975.

15

Maurel *et al* (1989) "Biotechnology of Plasma Proteins", Colloque INSERM **175**, 19-24.

Romanos, M. *et al* (1992) *Yeast* **8**, 423-488.

20

Rothstein, R. J. (1983) *Methods Enzymol.* **101**, 202-211.

Russo, P. *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 3671-3675.

25   Sambrook, J. *et al* (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sleep, D. *et al* (1991) *Bio/Technology* **9**, 183-187.

30   Toh-e *et al* (1993) *Yeast* **9**, 481-494.

20

Wang, H.Y. *et al* (1979) *Biotechnology & Bioeng.* **21**, 975

Yanisch-Perron, C. *et al* (1985) *Gene* **33**, 103-119.

5

21

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: Delta Biotechnology Limited
        (B) STREET: Castle Court, Castle Boulevard
        (C) CITY: Nottingham
        (E) COUNTRY: United Kingdom
        (F) POSTAL CODE (ZIP): NG7 1FD

    (ii) TITLE OF INVENTION: Yeast Strains

(iii) NUMBER OF SEQUENCES: 6

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: GB 9411356.0
        (B) FILING DATE: 07-JUN-1994

(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 40 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (ix) FEATURE:
        (A) NAME/KEY: misc_feature
        (B) LOCATION: 1..40
        (D) OTHER INFORMATION: /note= "Oligonucleotide for PCR
            amplification of 5' end of Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTATTTCCTA TTTCGGGAAT TCTTAAAGAC AAAAAAGCTC
        40

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (ix) FEATURE:
        (A) NAME/KEY: misc_feature
        (B) LOCATION: 1..28
        (D) OTHER INFORMATION: /note= "Oligonucleotide for PCR
           amplification of the 5' end of the Hsp150 gene."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGCTGTGGTG CTGCAGATGA TGCGCTGG
        28

(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

(ix) FEATURE:
    (A) NAME/KEY: misc_feature
    (B) LOCATION: 1..30
    (D) OTHER INFORMATION: /note= "Oligonucleotide for PCR
        amplification of 3' end of the Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCTACTTCCG CTTCTGCAGC CGCTACCTCC
  30

(2) INFORMATION FOR SEQ ID NO: 4:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 38 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: cDNA

  (iii) HYPOTHETICAL: NO

  (ix) FEATURE:
    (A) NAME/KEY: misc_feature
    (B) LOCATION: 1..38
    (D) OTHER INFORMATION: /note= "Oligonucleotide for PCR
        amplification of the 3' end of the Hsp150 gene."

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCCGTGTAGC GAGGGAATTC TGTGGTCACG ATCACTCG
  38

(2) INFORMATION FOR SEQ ID NO: 5:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 2048 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
    (A) ORGANISM: Saccharomyces cerevisiae

(ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION: 397..1638

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGTGATCTTA CTATTTCCTA TTTCGGAAAT TATTAAAGAC
AAAAAAGCTC ATTAATGGCT   60

TTCCGTCTGT AGTGATAAGT CGCCAACTCA GCCTAATTTT
TCATTTCTTT ACCAGATCAG   120

GAAAACTAAT AGTACAAATG AGTGTTTTCT CAAGCGGAAC
ACCACATTTT GAGCTAAATT   180

TAGATTTTGG TCAAAATAAG AAAGATCCTA AAAAAGGAAT
GGTTGGTGAA AAATTTATTA   240

GCTTGAATGG TAGGAATCCT CGAGATATAA AAGGAACACT
TGAAGTCTAA CGACAATCAA   300

TTTCGATTAT GTCCTTCCTT TTACCTCAAA GCTCAAAAAA
ATATCAATAA GAAACTCATA   360

TTCCTTTTCT AACCCTAGTA CAATAATAAT AATATA ATG CAA
TAC AAA AAG ACT   414
                          Met Gln Tyr Lys Lys Thr
                           1   5

TTG GTT GCC TCT GCT TTG GCC GCT ACT ACA TTG GCC GCC
TAT GCT CCA   462
Leu Val Ala Ser Ala Leu Ala Ala Thr Thr Leu Ala Ala Tyr Ala Pro
     10   15   20

TCT GAG CCT TGG TCC ACT TTG ACT CCA ACA GCC ACT TAC
AGC GGT GGT   510

25

Ser Glu Pro Trp Ser Thr Leu Thr Pro Thr Ala Thr Tyr Ser Gly·Gly
   25   30   35

GTT ACC GAC TAC GCT TCC ACC TTC GGT ATT GCC GTT CAA
CCA ATC TCC   558
Val Thr Asp Tyr Ala Ser Thr Phe Gly Ile Ala Val Gln Pro Ile Ser
 40   45   50

ACT ACA TCC AGC GCA TCA TCT GCA GCC ACC ACA GCC TCA
TCT AAG GCC   606
Thr Thr Ser Ser Ala Ser Ser Ala Ala Thr Thr Ala Ser Ser Lys Ala
 55   60   65   70

AAG AGA GCT GCT TCC CAA ATT GGT GAT GGT CAA GTC CAA
GCT GCT ACC   654
Lys Arg Ala Ala Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Ala Thr
     75   80   85

ACT ACT GCT TCT GTC TCT ACC AAG AGT ACC GCT GCC GCC
GTT TCT CAG   702
Thr Thr Ala Ser Val Ser Thr Lys Ser Thr Ala Ala Ala Val Ser Gln
    90   95   100

ATC GGT GAT GGT CAA ATC CAA GCT ACT ACT AAG ACT ACC
GCT GCT GCT   750
Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr Lys Thr Thr Ala Ala Ala
  105   110   115

GTC TCT CAA ATT GGT GAT GGT CAA ATT CAA GCT ACC ACC
AAG ACT ACC   798
Val Ser Gln Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr Lys Thr Thr
 120   125   130

TCT GCT AAG ACT ACC GCC GCT GCC GTT TCT CAA ATC AGT
GAT GGT CAA   846
Ser Ala Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Ser Asp Gly Gln
135   140   145   150

ATC CAA GCT ACC ACC ACT ACT TTA GCC CCA AAG AGC ACC
GCT GCT GCC   894
Ile Gln Ala Thr Thr Thr Thr Leu Ala Pro Lys Ser Thr Ala Ala Ala
   155   160   165

GTT TCT CAA ATC GGT GAT GGT CAA GTT CAA GCT ACC ACC

ACT ACT TTA   942
Val Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Thr Thr Leu
        170   175   180

GCC CCA AAG AGC ACC GCT GCT GCC GTT TCT CAA ATC GGT
GAT GGT CAA   990
Ala Pro Lys Ser Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln
        185   190   195

GTT CAA GCT ACT ACT AAG ACT ACC GCT GCT GCT GTC TTT
CAA ATT GGT   1038
Val Gln Ala Thr Thr Lys Thr Thr Ala Ala Ala Val Phe Gln Ile Gly
      200   205   210

GAT GGT CAA GTT CTT GCT ACC ACC AAG ACT ACT CGT GCC
GCC GTT TCT   1086
Asp Gly Gln Val Leu Ala Thr Thr Lys Thr Thr Arg Ala Ala Val Ser
215   220   225   230

CAA ATC GGT GAT GGT CAA GTT CAA GCT ACT ACC AAG ACT
ACC GCT GCT   1134
Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Lys Thr Thr Ala Ala
        235   240   245

GCT GTC TCT CAA ATC GGT GAT GGT CAA GTT CAA GCA ACT
ACC AAA ACC   1182
Ala Val Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Lys Thr
        250   255   260

ACT GCC GCA GCT GTT TCC CAA ATT ACT GAC GGT CAA GTT
CAA GCC ACT   1230
Thr Ala Ala Ala Val Ser Gln Ile Thr Asp Gly Gln Val Gln Ala Thr
        265   270   275

ACA AAA ACC ACT CAA GCA GCC AGC CAA GTA AGC GAT GGC
CAA GTC CAA   1278
Thr Lys Thr Thr Gln Ala Ala Ser Gln Val Ser Asp Gly Gln Val Gln
        280   285   290

GCT ACT ACT GCT ACT TCC GCT TCT GCA GCC GCT ACC TCC
ACT GAC CCA   1326
Ala Thr Thr Ala Thr Ser Ala Ser Ala Ala Ala Thr Ser Thr Asp Pro
295   300   305   310

27

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GTC GAT GCT GTC TCC TGT AAG ACT TCT GGT ACC TTA GAA
ATG AAC TTA     1374
Val Asp Ala Val Ser Cys Lys Thr Ser Gly Thr Leu Glu Met Asn Leu
       315         320         325


AAG GGC GGT ATC TTA ACT GAC GGT AAG GGT AGA ATT GGT
TCT ATT GTT     1422
Lys Gly Gly Ile Leu Thr Asp Gly Lys Gly Arg Ile Gly Ser Ile Val
       330         335         340


GCT AAC AGA CAA TTC CAA TTT GAC GGT CCA CCA CCA CAA
GCT GGT GCC     1470
Ala Asn Arg Gln Phe Gln Phe Asp Gly Pro Pro Pro Gln Ala Gly Ala
     345         350         355


ATC TAC GCT GCT GGT TGG TCT ATA ACT CCA GAC GGT AAC
TTG GCT ATT     1518
Ile Tyr Ala Ala Gly Trp Ser Ile Thr Pro Asp Gly Asn Leu Ala Ile
   360         365         370


GGT GAC AAT GAT GTC TTC TAC CAA TGT TTG TCC GGT ACT
TTC TAC AAC     1566
Gly Asp Asn Asp Val Phe Tyr Gln Cys Leu Ser Gly Thr Phe Tyr Asn
375         380         385         390


TTG TAC GAC GAA CAC ATT GGT AGT CAA TGT ACT CCA GTC
CAC TTG GAA     1614
Leu Tyr Asp Glu His Ile Gly Ser Gln Cys Thr Pro Val His Leu Glu
       395         400         405


GCT ATC GAT TTG ATA GAC TGT TAAGCAGAAA ACTATTAGTT
CTTTTATCCT     1665
Ala Ile Asp Leu Ile Asp Cys
       410


GATGACTTTT TCTCATTTGC ATTGATTAGA AAGGAAAAAA
AGAAGTGTCC TTTTCTACTA     1725


CTACTCTAGT CGCATCCATT CCTTTGCATT TATCTTTTCT
GCGGTTGGCC AATCCATTCT     1785


TCCGAGAATT TGGCTAGCCA TACTTGATGT TTTCCCATTA
TTGGTTCGTT TGGCAATGCT     1845
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AATTTTCTTA ATTGCCCCTT ATATACTCTT CCATAAAATG
TTTTTTTTAT AACTAATTTT   1905

CTGTATATCA TTATCTAATA ATCTTATAAA ATGTTAAAAA
GACTTGGAAA GCAACGAGTG   1965

ATCGTGACCA CATAATTGCC TCGCTACACG GCAAAAATAA
GCCAGTCCTA ATGTGTATAT   2025

TAAAGGCTGC ATGTGGCTAC GTC
2048

(2) INFORMATION FOR SEQ ID NO: 6:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 413 amino acids
         (B) TYPE: amino acid
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Gln Tyr Lys Lys Thr Leu Val Ala Ser Ala Leu Ala Ala Thr Thr
 1   5   10   15

Leu Ala Ala Tyr Ala Pro Ser Glu Pro Trp Ser Thr Leu Thr Pro Thr
        20   25   30

Ala Thr Tyr Ser Gly Gly Val Thr Asp Tyr Ala Ser Thr Phe Gly Ile
       35   40   45

Ala Val Gln Pro Ile Ser Thr Thr Ser Ser Ala Ser Ser Ala Ala Thr
    50   55   60

Thr Ala Ser Ser Lys Ala Lys Arg Ala Ala Ser Gln Ile Gly Asp Gly
 65   70   75   80

Gln Val Gln Ala Ala Thr Thr Thr Ala Ser Val Ser Thr Lys Ser Thr
            85   90   95

Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr
        100   105   110

29

Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Ile Gln
     115   120   125

Ala Thr Thr Lys Thr Thr Ser Ala Lys Thr Thr Ala Ala Ala Val Ser
   130   135   140

Gln Ile Ser Asp Gly Gln Ile Gln Ala Thr Thr Thr Thr Leu Ala Pro
145   150   155   160

Lys Ser Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Val Gln
     165   170   175

Ala Thr Thr Thr Thr Leu Ala Pro Lys Ser Thr Ala Ala Ala Val Ser
     180   185   190

Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Lys Thr Thr Ala Ala
     195   200   205

Ala Val Phe Gln Ile Gly Asp Gly Gln Val Leu Ala Thr Thr Lys Thr
   210   215   220

Thr Arg Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr
225   230   235   240

Thr Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Val
     245   250   255

Gln Ala Thr Thr Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Thr Asp
     260   265   270

Gly Gln Val Gln Ala Thr Thr Lys Thr Thr Gln Ala Ala Ser Gln Val
     275   280   285

Ser Asp Gly Gln Val Gln Ala Thr Thr Ala Thr Ser Ala Ser Ala Ala
   290   295   300

Ala Thr Ser Thr Asp Pro Val Asp Ala Val Ser Cys Lys Thr Ser Gly
305   310   315   320

Thr Leu Glu Met Asn Leu Lys Gly Gly Ile Leu Thr Asp Gly Lys Gly
     325   330   335

Arg Ile Gly Ser Ile Val Ala Asn Arg Gln Phe Gln Phe Asp Gly Pro
     340   345   350

30

Pro Pro Gln Ala Gly Ala Ile Tyr Ala Ala Gly Trp Ser Ile Thr Pro
        355   360   365

Asp Gly Asn Leu Ala Ile Gly Asp Asn Asp Val Phe Tyr Gln Cys Leu
    370   375   380

Ser Gly Thr Phe Tyr Asn Leu Tyr Asp Glu His Ile Gly Ser Gln Cys
385   390   395   400

Thr Pro Val His Leu Glu Ala Ile Asp Leu Ile Asp Cys
        405   410

CLAIMS

1. A process for preparing a desired protein from yeast, comprising culturing the yeast and obtaining the desired protein, characterised in that the yeast is deficient in heat shock protein 150 (Hsp150).

2. A process according to Claim 1 wherein the yeast has a defect in its genome such that a reduced level of the Hsp150 protein is produced.

3. A process according to Claim 2 wherein substantially no Hsp150 protein is produced.

4. A process according to any one of the preceding claims wherein the desired protein is an albumin.

5. A process according to Claim 4 wherein the desired protein is a human albumin.

6. A process according to any one of the preceding claims wherein the yeast is a *Torulaspora, Kluyveromyces, Schizosaccharomyces, Pichia* or *Saccharomyces* species.

7. A process according to Claim 6 wherein the yeast is *S. cerevisiae*.

8. A process according to any one of the preceding claims wherein the desired protein is secreted from the yeast into the surrounding medium and purified therefrom.

9. A protein prepared by a process according to any one of the preceding claims.

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10. A culture medium containing a desired protein and prepared by a process according to any one of Claims 1 to 7.

11. A yeast transformed to express a desired protein which will co-purify with Hsp150 in chromatographic techniques, characterised in that the yeast is deficient in Hsp150.

12. A yeast according to Claim 11 wherein the yeast has a defect in its genome such that a reduced level of the Hsp150 protein is produced.

13. A yeast according to Claim 11 wherein substantially no Hsp150 protein is produced by the yeast.

14. A yeast according to any one of Claims 11 to 13 wherein the desired protein is an albumin.

15. A yeast according to Claim 14 wherein the desired protein is a human albumin.

16. A yeast according to any one of Claims 11 to 15 wherein the yeast is *Torulaspora*, *Kluyveromyces*, *Schizosaccharomyces* or *Saccharomyces* species.

17. A yeast according to Claim 16 wherein the yeast is *S. cerevisiae*.

18. A yeast according to any one of Claims 11 to 17 wherein the yeast is transformed with a DNA construct such that the desired protein is secreted from the yeast during culturing thereof.

19. A method of preparing a yeast according to any one of Claims 11 to 18

33

comprising the steps of

(i)   transforming the yeast with a coding sequence for expression of the desired protein, and

(ii)   disrupting the genome of the yeast such that the yeast has an abnormally low level of Hsp150,

wherein steps (i) and (ii) may be carried out in either order or simultaneously.

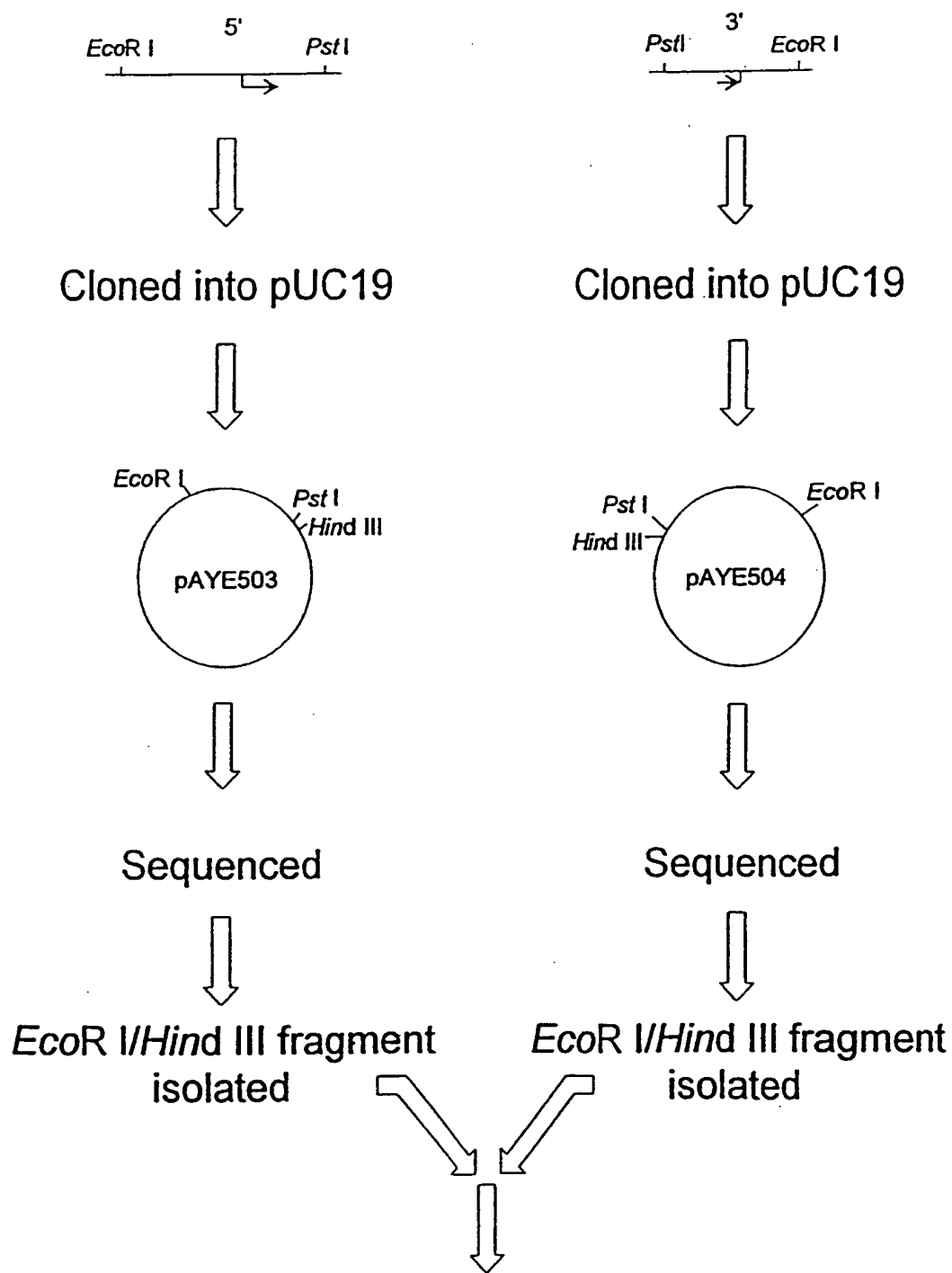## 5' and 3' regions of *HSP150* gene obtained by PCR:

Figure 1

Figure 1 cont'd
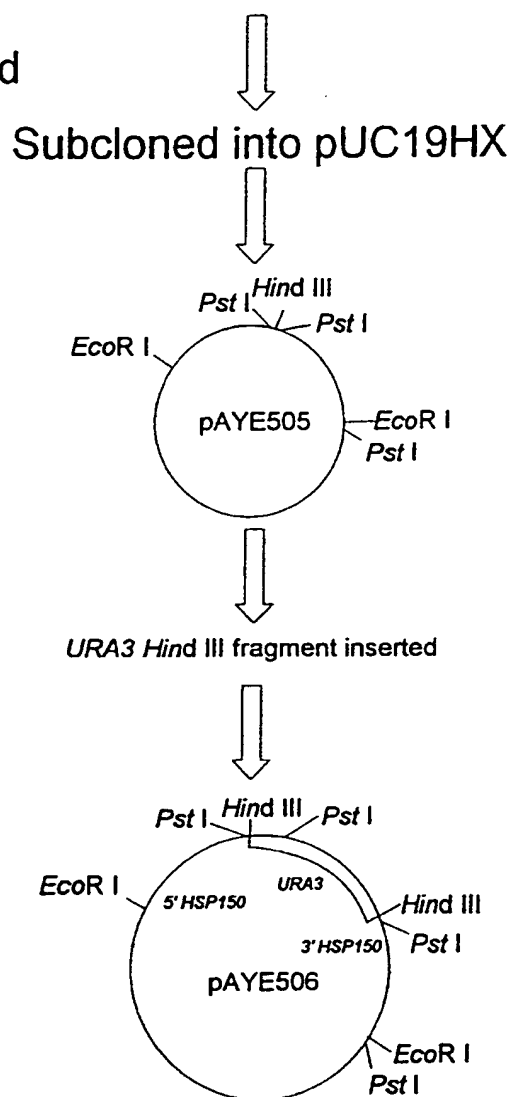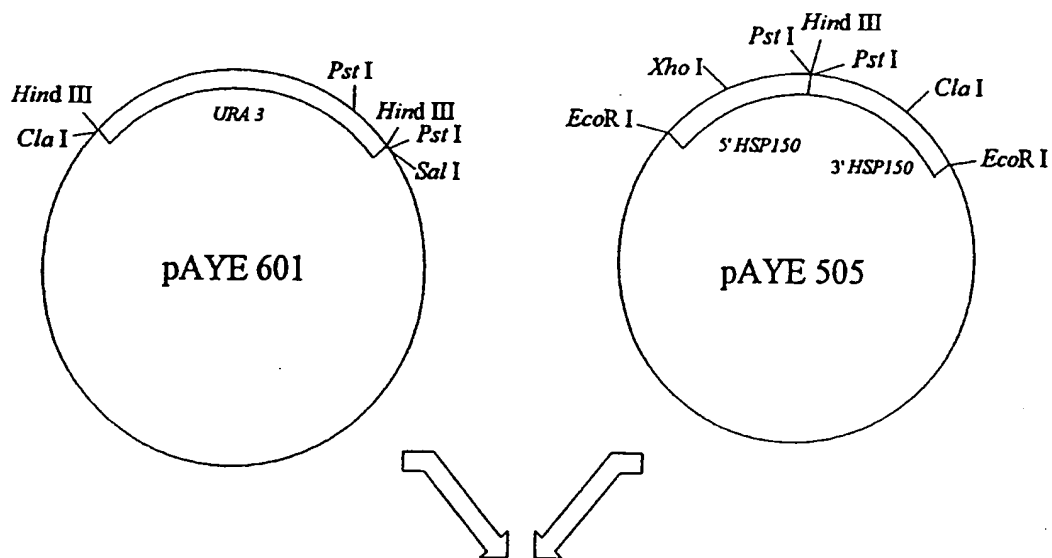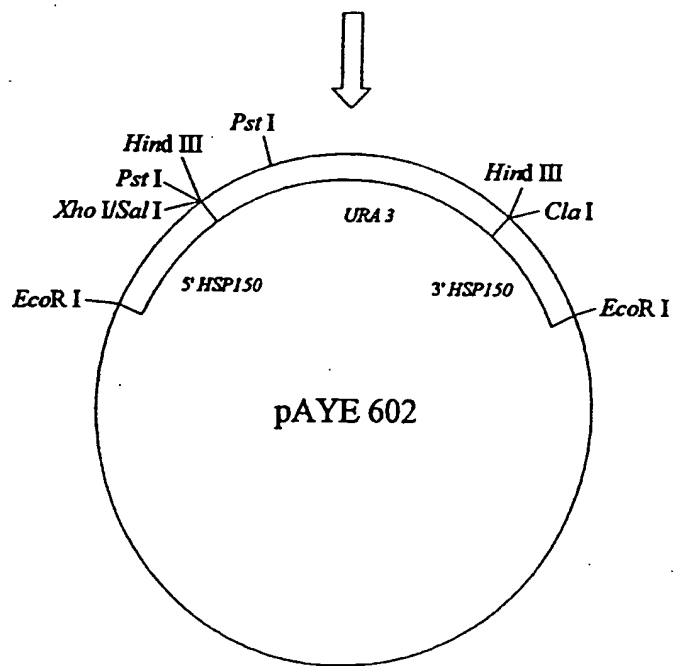
Fig 2

Ligate *Cla* I/ *Sal* I *URA 3* fragment into *Cla* I/*Xho* I cut pAYE 505

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/14   C12N1/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOL GEN GENET, MAY 1993, 239 (1-2) P273-80, GERMANY, RUSSO P ET AL 'Dual regulation by heat and nutrient stress of the yeast HSP150 gene encoding a secretory glycoprotein.' see the whole document	1,2, 6-12, 16-19
X	BIOTECHNOLOGY, vol. 8, 1990 NEW YORK US, pages 42-46, SLEEP D. ET AL. 'The secretion of human serum albumin from the yeast Sachcharomyces cerevisiae using five different leader sequences' cited in the application see the whole document	9,10

☐ Further documents are listed in the continuation of box C.   ☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 1995	10.10.95

Name and mailing address of the ISA	Authorized officer
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